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ORIGINAL ARTICLE

Presence of aggregates of smooth endoplasmic reticulum in MII oocytes affects oocyte competence: molecular-based evidence

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STUDY QUESTION: Does the presence of aggregates of smooth endoplasmic reticulum (SERa) impact the transcriptome of human metaphase II (MII) oocytes?.

SUMMARY ANSWER: The presence of SERa alters the molecular status of human metaphase II oocytes.

WHAT IS KNOWN ALREADY: Oocytes presenting SERa are considered dysmorphic. Oocytes with SERa (SERa+) have been associated with reduced embryological outcome and increased risk of congenital anomalies, although some authors have reported that SERa+ oocytes can lead to healthy newborns. The question of whether or not SERa+ oocytes should be discarded is still open for debate, and no experimental information about the effect of the presence of SERa on the oocyte molecular status is available.

STUDY DESIGN, SIZE, DURATION: This study included 28 women, aged <38 years, without any ovarian pathology, and undergoing IVF treatment. Supernumerary MII oocytes with no sign of morphological alterations as well as SERa+ oocytes were donated after written informed consent. A total of 31 oocytes without SERa (SERa−) and 24 SERa+ oocytes were analyzed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Pools of 8–10 oocytes for both group were prepared. Total RNA was extracted from each pool, amplified, labeled and hybridized on oligonucleotide microarrays. Analyses were performed by R software using the limma package.

MAIN RESULTS AND THE ROLE OF CHANCE: The expression profiles of SERa+ oocytes significantly differed from those of SERa− oocytes in 488 probe sets corresponding to 102 down-regulated and 283 up-regulated unique transcripts. Gene Ontology analysis by DAVID bioinformatics disclosed that genes involved in three main biological processes were significantly down-regulated in SERa+ oocytes respective to SERa− oocytes: (i) cell and mitotic/meiotic nuclear division, spindle assembly, chromosome partition and G2/M transition of mitotic cell cycle; (ii) organization of cytoskeleton and microtubules; and (iii) mitochondrial structure and activity. Among the transcripts up-regulated in SERa+ oocytes, the most significantly ($P = 0.002$) enriched GO term was 'GoLoco motif', including the *RAP1GAP*, *GPSM3* and *GPSM1* genes.

LARGE SCALE DATA: Raw microarray data are accessible through GEO Series accession number GSE106222 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106222).

LIMITATIONS, REASONS FOR CAUTION: Data validation in a larger cohort of samples would be beneficial, although we applied stringent criteria for gene selection (fold-change >3 or <1/3 and FDR < 0.1). Surveys on clinical outcomes, malformation rates and follow-up of babies born after transfer of embryos from SERa+ oocytes are necessary.

WIDER IMPLICATIONS OF THE FINDINGS: We provide information on the molecular status of SERa+ oocytes, highlighting possible associations between presence of SERa, altered oocyte physiology and reduced developmental competence. Our study may offer further information that can assist embryologists to make decisions on whether, and with what possible implications, SERa+ oocytes should be used.

We believe that the presence of SERa should be still a 'red flag' in IVF practices and that the decision to inseminate SERa+ oocytes should be discussed on a case-by-case basis.

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Key words: smooth endoplasmic reticulum aggregates / oocyte dysmorphism / oocyte physiology / microarray / gene expression

Introduction

Smooth endoplasmic reticulum (SER) is one of the most common organelles in the ooplasm. It is an interconnected network of membrane-enclosed sacs or tubules, whose most important function in oocyte physiology is storage and redistribution of calcium. During the fertilization process, sperm entry into the oocyte triggers waves of intracellular free calcium as the result of a series of release and re-uptake of calcium stored in SER. Calcium release is primarily mediated by the phosphoinositide pathway of the oocyte, presumably induced by the activation of phospholipase C by a tyrosine kinase activated by the oocyte receptor Juno after interaction with Izumo 1 sperm protein (Bianchi *et al.*, 2014). The phospholipase C causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Another hypothesis is that the soluble phospholipase C isoform zeta (PLCζ) diffuses from the sperm into the ooplasma upon sperm–oocyte fusion and starts cleavage of PIP_2 (Saunders *et al.*, 2002). In both models, the signaling cascade ends with the binding of IP_3 to its receptors on the surface of the SER and the subsequent induction of calcium release.

After ovarian stimulation, a certain proportion of oocytes present cytoplasmic or extra-cytoplasmic abnormal morphological traits, including non-spherical shape, cytoplasmic irregularities, first polar body fragmentation and thick or dark zona pellucida (Rienzi *et al.*, 2012; ESHRE Atlas of Human Embryology, http://atlas.eshre.eu). Aggregates of SER (SERa) in the ooplasm is one of the cytoplasmic dysmorphisms of oocytes. These aggregations appear as round flat disks in the ooplasma and they correspond to large clusters of tubular SER surrounded by mitochondria and by dense granules containing tiny vesicles (Sá *et al.*, 2011). They arise by dilatation and fusion of SER during oocyte maturation and mostly disappear after pronuclei appearance (Otsuki *et al.*, 2004). Under inverted microscopy, SERa can be morphologically distinguished from fluid-filled vacuoles, since they appear as single or multiple translucent vacuole-like structures of 10–80 μm of diameter (Ebner *et al.*, 2008). The SERa are associated with cytoskeleton alterations including increased spindle length and cortical actin disorganization (Dal Canto *et al.*, 2017). The etiology of such a dysmorphism is not known. The formation of SERa is not related to patient age, number of retrieved oocytes, presence of endometriosis in ovaries or thickness of the endometrium (Otsuki *et al.*, 2004). However, it was found that SERa are associated with doses and duration of gonadotropin administration (Ebner *et al.*, 2008). Moreover, a genetic predisposition cannot be excluded, since SERa in all retrieved oocytes of the same patient and repetitive presence of SERa from one cycle to another have been noticed (Meriano *et al.*, 2001; Akarsu *et al.*, 2009).

SERa are supposed to impact on oocyte physiology causing abnormal calcium signals during oocyte activation, abnormal mitochondrial respiratory activity, possible chromosome segregation errors and abnormal cytokinesis (Van Blerkom, 2011; Dal Canto *et al.*, 2017). Overall, SERa may negatively affect fertilization and post-fertilization events, with implications for embryo quality, implantation and fetal development. For these reasons, the impact of SERa on embryological and obstetric outcomes has been evaluated. Some authors have reported that use of oocytes showing SERa (SERa+) results in significantly lower fertilization, embryo cleavage, blastocyst and pregnancy rates compared to controls (Sá *et al.*, 2011; Hattori *et al.*, 2014). Moreover, the presence of SERa+ oocytes or their use in IVF has been associated with malformations or genetic abnormalities in the newborns such as Beckwith–Wiedmann syndrome, diaphragmatic hernia, multiple malformations and cardiovascular defect (Otsuki *et al.*, 2004; Ebner *et al.*, 2008; Akarsu *et al.*, 2009). In 2011, based on these data, the Alpha Scientists and ESHRE guidelines advised against the use of SERa+ oocytes in IVF (Alpha Scientists in Reproductive Medicine and ESI, 2011). However, some authors have shown that healthy babies can be derived from SERa+ oocytes (Mateizel *et al.*, 2013; Hattori *et al.*, 2014). As a consequence, policies of IVF centers toward SERa+ oocytes are not homogeneous (Restelli *et al.*, 2015; Van Beirs *et al.*, 2015; Shaw-Jackson *et al.*, 2016) and the consensus recommendation not to inject/inseminate SERa+ oocytes may need to be revisited. In the meantime, it is the opinion of an Expert Panel that the decision to use SERa+ oocytes in IVF cycles should be discussed by the clinical team on a case-by-case basis (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Certainly, the exclusion of SERa+ oocytes from IVF cycles may cause an increased frequency of transfer cancellations (Restelli *et al.*, 2015), which can be perceived as an extremely negative outcome and couples might be discouraged about continuing ART treatment. Larger studies focused on correlating frequency and size of SERa with clinical outcomes and malformation rates, as well as follow-up of children, are mandatory. Moreover, no experimental data about the effect of the presence of SERa on the oocyte molecular profile are available to date.

The present study was aimed at analyzing, for the first time, the transcriptome of SERa+ oocytes. For this purpose, we compared the gene expression profiles of human metaphase II (MII) SERa+ oocytes with normal MII oocytes without SERa (SERa−) by using a microarray approach. This article represents a significant step toward a more objective evaluation of the impact of SER dysmorphism, offering evidence that can assist embryologists to more consciously and rationally decide whether, and with what possible risks, SERa+ oocytes should be used.

Materials and Methods

Patients selection, treatment and oocyte collection

This study included 28 women, undergoing IVF treatment, who were <38 years old (range: 25–38 years) and without any ovarian pathology (e.g. endometriosis, polycystic ovaries, ovarian insufficiency) to avoid any bias.

Standardovarian stimulation protocols were applied as previously described (Stigliani *et al.*, 2015). Cumulus–oocyte complexes were collected 34–36 h later by vaginal puncture under ultrasound echo-guidance, washed in Sydney IVF Gamete buffer (Cook Medical, Bloomington, IN, USA) and immediately incubated in Sydney IVF Fertilization medium (Cook Medical) at 37°C in a humidified atmosphere of 6% CO_2, 5% O_2 using Galaxy 48 R incubators (New Brunswick Scientific, Edison, NJ, USA). After 2–4 h, oocytes were denuded of cumulus cells by enzymatic treatment with 80 IU/ml hyaluronidase solution (Origio, Målov, Denmark) and placed in Sydney IVF Cleavage medium (Cook Medical). Supernumerary mature SERa− and SERa+ oocytes were frozen in sterile, DNA-, DNase-, RNase-, PCR inhibitors-free tubes (Eppendorf, Hamburg, Germany) containing QIAzol® Lysis Reagent (miRNeasy Micro Kit, Qiagen, Hilden, Germany) and stored at −80°C until RNA purification.

Ethical approval

Our center scrupulously follows the recommendations of the Alpha/ESHRE consensus and SERa+ oocytes are systematically discarded in ICSI procedures. Supernumerary mature SERa− and SERa+ oocytes were donated after written informed consent. The study was approved by the Ethical Committee of Regione Liguria (approval no. 196REG2017).

Study design

Oocytes were randomly divided into pools for each group in order to minimize any patient-specific variability in gene expression. We prepared three pools of normal MII oocytes ('SERa−' group: a total of 31 oocytes from 13 patients in two pools of 10 and one pool of 11 oocytes), and three pools of MII oocytes with SERa ('SERa+' group: a total of 24 oocytes from 15 patients in pools of 8 oocytes each) (Supplementary Fig. S1). There was no difference in patients' age (mean ± SD) between the two groups (SERa−: 34.2 ± 3.6 years; SERa+: 32.4 ± 4.2 years) and among the pools (SERa− pool 1: 34.6 ± 3.1 years; SERa− pool 2: 34.8 ± 2.8 years; SERa− pool 3: 33.0 ± 3.1 years; SERa+ pool 1: 32.3 ± 4.5; SERa+ pool 2: 33.9 ± 4.2; SERa+ pool 3: 33.1 ± 4.5).

Moreover, between the two groups, there were no differences in average (mean ± SD) of total doses gonadotropins (SERa−: 2263 IU ± 1399; SERa+: 2536 IU ± 1378) and final serum concentrations of 17-beta-estradiol (SERa−: 1716 ng/L ± 948; SERa−: 1597 ng/L ± 1037).

RNA isolation and quantitation

Total RNA was extracted from each pool of oocytes using the miRNeasy Micro kit (Qiagen), according to the manufacturer's procedure. Quantification and quality control of RNA were performed by RNA 6000 Pico kit on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Gene expression profiling

Total RNA from each pool (5ng) was amplified using Ovation Pico WTA System V2 (NuGEN Technologies, San Carlos, CA, USA), which is an accurate and reliable technique in profiling non-coding and coding genes by PCR and microarray (Kurn *et al.*, 2005; Vermeulen *et al.*, 2009; Scaruffi *et al.*, 2010; https://www.nugen.com/sites/default/files/AN_1v2.1-Agilent_Solution_FFPE_WTA.pdf). After amplification, cDNA was labeled by Enzymatic Labeling Kit (Agilent Technologies). Purified-Cye3-labeled cDNA (3 µg) were hybridized to Human GE 4×44 K v2 microarrays (Agilent Technologies) and processed as previously described (Stigliani *et al.*, 2015). The raw and normalized data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE106222 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106222).

Statistical analysis of microarray data

Tab-delimited text files containing FE results were acquired and analyzed as described elsewhere (Stigliani *et al.*, 2015). Differential expression analysis between groups was carried out using Student's *t*-test (unpaired, two-tailed, unequal variance). Probes with False Discovery Rate (FDR) < 0.1 and fold-change between the two groups larger than three were selected as differentially expressed genes. Agglomerative hierarchical clusters were computed according to the Ward method (Ward, 1963) and using the Euclidean distance between vectors of log 2-transformed expression measures.

Gene ontology analysis

DAVID 6.8 software (http://david.abcc.ncifcrf.gov; Dennis *et al.*, 2003) was used to determine biological pathways significantly ($P < 0.05$) over-represented in our datasets.

Quantitative PCR (qPCR)

Expression analysis was performed using specific TaqMan® assays (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate in a Mastercycler epRealPlex^2 S system (Eppendorf). Negative controls (water as template) were run simultaneously. RealPlex software v. 2.0 (Eppendorf) was used to determine quantification cycle (Cq). The expression of each target gene relative to the reference *HPRT1* gene (Santonocito *et al.*, 2013), $\Delta Cq = (Cq_{target\ gene} - Cq_{HPRT1})$ was calculated. The equation $2^{-\Delta\Delta Cq}$ was used to normalize the expression of each target in SERa+ oocytes versus SERa− oocytes. The expression levels of each target gene measured in SERa+ respect to SERa− oocytes were compared by two-tailed Student's *t*-test (MedCalc® software, Mariakerke, Belgium).

Results

In all samples processed in the present study, the RNA amount was suitable to be amplified, labeled and hybridized on microarrays. Regarding microarray analyses, the expression dataset initially contained 44 495 probes and six samples divided into two groups: three pool samples in the SERa+ group and three pool samples in the SERa− group.

After filtering and condensing by replicates average and control spots removal, the expression dataset contained 23 574 probes. Background corrected intensities of duplicate spots on each array were averaged and on average each array contained 16 852 probes.

The expression profiles of SERa+ oocytes differed significantly from those of SERa− oocytes in 488 probe sets corresponding to 102 down-regulated and 283 up-regulated unique transcripts (Fig. 1, Supplementary Tables SI and SII). The remaining 103 probe sets were excluded because they were unknown or non-coding sequences.

To get an insight into potential functional roles, the genes differentially expressed by SERa+ and SERa− oocytes were searched in the Gene Ontology (GO) annotations. As shown in Table I, genes involved in three main biological processes were significantly down-regulated in

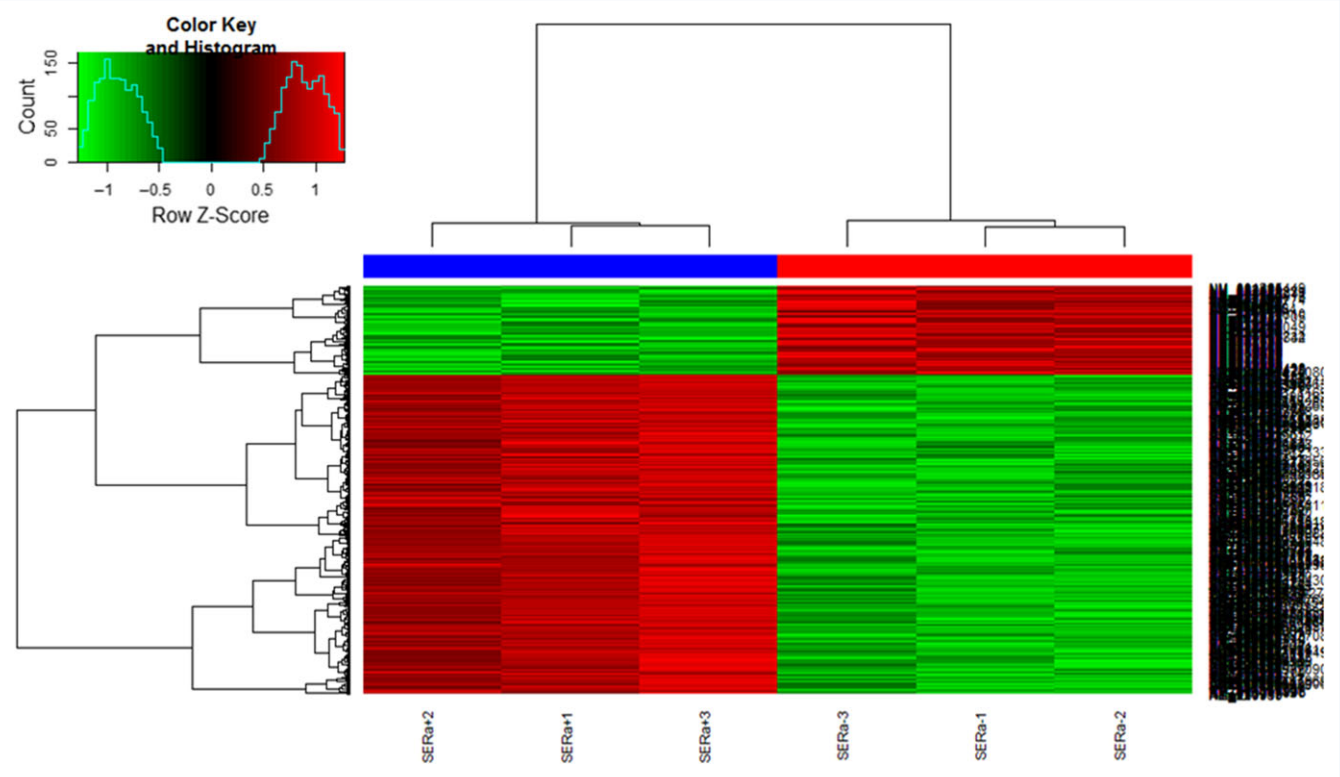

Figure 1 Agglomerative hierarchical clustered heat map of differentially expressed probe sets in SERa+ (samples in blue) versus SERa− (samples in red) MII oocytes. Each color patch represents the expression level of transcripts (row) in that sample (column), with a continuum of expression levels from bright green (lowest) to bright red (highest).

Table I The most significantly ($P < 0.05$) enriched Gene Ontology Biological Process (GOBP) terms in genes down-regulated in SERa+ oocytes versus SERa− oocytes.

GOBP term/Keywords	*P* value	Gene symbol
GO:0007067~mitotic nuclear division	0.008	*NEK2, CENPV, BIRC5, HAUS8, CENPX, RPS3, MAU2*
GO:0051301~cell division	0.008	*MAU2, NEK2, CENPV, BIRC5, HAUS8, CENPX, RPS3, KLLN, MCM7, CROCC*
GO:0051225~spindle assembly	0.008	*NEK2, HAUS8, RPS3*
GO:0000777~condensed chromosome kinetochore	0.010	*NEK2, CENPV, BIRC5, CENPX*
GO:0000086~G2/M transition of mitotic cell cycle	0.018	*MAU2, NEK2, BIRC5, HAUS8*
Chromosome partition		
Cytoskeleton	0.014	*MICALL2, FGD1, DNAI1, TLN2, CROCC, NEK2, MAP2, CENPV, BIRC5, HAUS8, WDR34, RPS3*
Microtubule	0.039	*DNAI1, NEK2, MAP2, BIRC5, HAUS8*
Mitochondrial structure	0.031	*SDHA, MRPL11, ALKBH7, COX14, NDUFA3, NUDT1, COA7, FDXR, SLC25A26, GSTP1, RPS3*
Mitochondrial activity	0.013	*SDHA, NDUFA3, FDXR, FADS2*

SERa+ oocytes respect to SERa− oocytes: (i) cell and mitotic/meiotic nuclear division, mitotic/meiotic spindle assembly and chromosome partition, and G2/M transition of cell cycle (*NEK2*, *CENPV*, *BIRC5*, *HAUS8*, *CENPX*, *RPS3*, *MAU2*, *KLLN*, *MCM7*, *CROCC*); (ii) organization of cytoskeleton and microtubules (*MICALL2*, *FGD1*, *DNAI1*, *TLN2*, *CROCC*, *NEK2*, *MAP2*, *CENPV*, *BIRC5*, *HAUS8*, *WDR34*, *RPS3*); (iii) mitochondrial structure and activity (*SDHA*, *MRPL11*, *ALKBH7*, *COX14*, *NDUFA3*, *NUDT1*, *COA7*, *FDXR*, *SLC25A26*, *GSTP1*, *RPS3*, *FADS2*).

As regarding the 283 transcripts up-regulated in SERa+ oocytes, the most significantly ($P = 0.002$) enriched GO term was 'GoLoco motif', including the *RAP1GAP*, *GPSM3* and *GPSM1* genes. In heterotrimeric G-proteins, the GoLoco motif consists of three small alpha helices with a highly conserved Asp–Gln–Arg triad. It participates directly in

GDP binding. Intriguingly, GPSM1 (G-protein-signaling modulator 1), is localized in endoplasmic reticulum membrane of Golgi apparatus, and controls spindle orientation, and RAP1GAP (RAP1 GTPase Activating Protein) is involved in cell proliferation, differentiation and embryogenesis processes.

Since SER is an important component in calcium signaling, we also looked for differently expressed genes that could have implications on calcium signaling. We found that in SERa+ oocytes, 4 down-regulated (*FAT1*, *ITGA10*, *LMAN2* and *TGM4*) and 7 up-regulated (*DLK1*, *DSPP*, *EYS*, *MMP28*, *PCDHB13*, *PCDHB8* and *PCDHGA12*) genes encode proteins which bind at least one calcium atom or proteins whose function is calcium-dependent.

Finally, based on the limited amount of cDNA available, we validated expression levels of the following significantly differentially expressed targets, chosen according to their function: *CROCC*, *HAUS8*, *RPS3* (genes involved in cell and mitotic/meiotic nuclear division); *MAP2* (organization of cytoskeleton and microtubules); *MRPL11*, *COA7*, *FDXR* (mitochondrial structure and activity); and *RAP1GAP*, *GPSM3*, *GPSM1* ('GoLoco motif' including genes). Microarray data were confirmed for six down-regulated genes (*CROCC*, *FDXR*, *HAUS8*, *MAP2*, *MRPL11*, *RPS3*) and the differences were significant for four of these genes (Table II). Failure in validating the remaining four genes may be due to their low level of expression.

Discussion

Ovarian stimulation recruits oocytes of different qualities, mostly due to asynchronous nuclear and cytoplasmic maturation (Ebner *et al.*, 2006). Based on current evidence, it has been agreed that non-homogeneous cytoplasm may represent normal variability among oocytes rather than a 'dysmorphism' with significance for IVF outcome (Alpha Scientists in Reproductive Medicine and ESI, 2011). The only exception is the appearance of SERa within the ooplasm. In 2011, based on data showing detrimental effects of this feature on fertilization, embryo development, implantation and clinical and newborn outcomes (Otsuki *et al.*, 2004; Ebner *et al.*, 2008; Akarsu *et al.*, 2009; Sá *et al.*, 2011; Hattori *et al.*, 2014), an Expert Panel strongly recommended that SERa+ oocytes should not be inseminated (Alpha Scientists in Reproductive Medicine and ESI, 2011). However, on this issue, there are contrasting reports in literature, since births of normal healthy babies derived from SERa+ oocytes have been reported (Mateizel *et al.*, 2013; Hattori *et al.*, 2014). Therefore, the questions of whether or not oocytes affected by the SER dysmorphism should be discarded and whether or not the ESHRE consensus regarding the use of SERa+ oocytes in IVF cycles should be reviewed are still open for debate. In the meantime, it is opinion of an Expert Panel that the decision to inject SERa+ oocytes should be reviewed by the clinical team on a case-by-case basis (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017).

Table II Selected genes differentially expressed in SERa+ oocytes as compared with SERa− oocytes.

	Fold change*	*P*-value*
Up-regulated genes		
GPSM1	1×10^{-2}	n.s.
GPSM3	5×10^{-2}	n.s.
RAP1GAP	3×10^{-4}	0.0073
Down-regulated genes		
COA7	1.1	n.s.
CROCC	0.2	n.s.
FDXR	6×10^{-4}	0.0400
HAUS8	6×10^{-2}	0.0016
MAP2	2×10^{-5}	<0.0001
MRPL11	2×10^{-3}	n.s.
RPS3	6×10^{-2}	0.0209

*Results obtained by qPCR analysis.

The aim of the present study was to investigate, for the first time, the effect of the presence of SERa on the molecular status of oocytes and thus to evaluate whether this feature reflects a compromised biological competence of the oocytes.

Our microarray analysis demonstrated that the gene expression profile of MII oocytes with SERa was significantly different respect to that of normal MII oocytes. One set of genes down-regulated in SERa+ oocytes were genes involved in cytokinesis and mitotic/meiotic regulation, spindle assembly and chromosome partition. Specifically, low expression levels of *NEK2* and *CROCC* genes may alter the centrosome cycle in which centrosome duplication and separation take place. In fact, NEK2 is a serine/threonine-protein kinase that localizes to the centrosome in mitosis. It is undetectable in the G1 phase, but starts accumulating during the S phase, reaching maximal levels in late G2 phase. In mitotic cells, NEK2 regulates centrosome disjunction, bipolar spindle formation and high-fidelity chromosome separation, by phosphorylating centrosome linker components such as beta-catenin and Rootletin (CROCC), resulting in their displacement from the centrosomes (Bahe *et al.*, 2005; Bahmanyar *et al.*, 2008). NEK2 is also involved in control of chromatin condensation during the first meiotic division. We hypothesize that low NEK2 activity in SERa+ oocytes may induce extra-centrosomal structures and chromosomal instability, as reported in pre-cancerous lesions and many tumours (D'Assoro *et al.*, 2002; Pihan *et al.*, 2003). In addition to directly contributing to centrosome amplification, altered NEK2 activity may lead to spindle multipolarity through impaired inhibition of supernumerary centrosomes clusters, an intrinsic cellular protection mechanism against centrosome amplification in mitosis (Milunovic-Jevtic *et al.*, 2016; Quintyne *et al.*, 2005).

Also *HAUS8*, *MAU2* and *BIRC5* genes are vital to mitotic spindle assembly and normal progression through mitosis, since they encode microtubule-binding complex proteins and components of the chromosome passage protein complex (CPC) (Uehara *et al.*, 2009; Watrin *et al.*, 2006). Therefore, in SERa+ oocytes, the reduced expression of *HAUS8*, *MAU2* and *BIRC5* may alter microtubule generation within the mitotic spindle, sister chromatid cohesion, chromosome alignment and segregation and cytokinesis.

In SERa+ oocytes, formation of a functional kinetochore outer plate may be negatively affected by the deregulation of *NEK2*, *CENPX* and *CENP-V* genes. The kinetochore outer plate is a very dynamic structure which is essential for kinetochore-microtubule attachment to form the spindle, which serves to join and move the sister chromatids of duplicated chromosomes to the metaphase plate during mitosis and meiosis (Tadeu *et al.*, 2008; Nishino *et al.*, 2012; Dornblut *et al.*, 2014). Maintenance of this chromatid-spindle cohesion is critical for

high-fidelity chromosome transmission. For example, it has been demonstrated that CENP-V depletion leads to misalignment of chromosomes in metaphase, chromosome mislocalization in anaphase, failure of cytokinesis and cell death, due to destabilization of the CPC (Tadeu et al., 2008). Thus, a possible consequence of depletion of *NEK2*, *CENPX* and *CENP-V* in SERa+ oocytes may be impairment of resolution of meiotic recombination intermediates by alteration of cleavage and rejoining of intermediates, such as Holliday junctions (Liu and West, 2004).

In SERa+ oocyte-derived embryos, the machinery of cell division may be destabilized also by up-regulation of three genes encoding for GoLoco (otherwise known as GPR or 'G-protein regulatory') motif proteins (Siderovski et al., 1999): *GPSM1*, *GPSM3* and *RAP1GAP*. The GoLoco proteins are a family of GTP-ase-activating proteins for heterotrimeric G-protein alpha subunits (Takesono et al., 1999). One subset of GoLoco proteins includes the R12 subfamily of RGS proteins (RGS12, RGS14 and the Drosophila ortholog Loco). A second one consists of the G-protein signaling modulator protein, GPSM1. A third subfamily includes GPSM3 and GPSM4. Rap1GAP is the only member of the fourth subset of GoLoco proteins. The majority of GoLoco motif-containing proteins bind to GDP-bound Gα subunits of the adenylyl-cyclase inhibitors and act as guanine nucleotide dissociation inhibitors (GDIs), slowing down the exchange of GDP for GTP and inhibiting association with Gβγ subunits (Willard et al., 2004). The GoLoco motif-containing proteins control mitotic spindle organization, microtubule interactions and chromosomal segregation during cell division (Willard et al., 2004). Therefore, altered expression levels of *GPSM1*, *GPSM3* and *RAP1GAP* genes in SERa+ oocytes may negatively influence the interaction between Gα and tubulin controlling microtubule force generation which is critical to regulating chromosomal segregation during cell division and spindle orientation. In fact, oocyte meiotic spindles are oriented perpendicular to the cortex at anaphase. During female meiosis, such an asymmetrically positioned meiotic spindle expels chromosomes into polar bodies, thus preserving the volume and contents of the oocyte (Fabritius et al., 2011). The one-pole spindle positioning plays an important role not only during oocyte meiosis, but may have important consequences also on cleavage events after SERa+ oocyte fertilization, and later on embryo development. In fact, the orientation of the mitotic spindle is known to be important in directing asymmetric cell divisions (Strome, 1993) that precede cellular differentiation during embryogenesis (Guo and Gao, 2009).

Another two putative actors in destabilization of cell cycle control in SERa+ oocytes are *KLLN* and *MCM7* that are involved in the S phase checkpoint (Qiao et al., 2015) and start of genome replication (Tsao et al., 2004), respectively.

In SERa+ oocytes, the reduced expression of genes involved in organization of cytoskeleton and microtubules confirms, at the molecular level, recent findings about alterations in spindle size and cortical actin disorganization associated to SER aggregates (Dal Canto et al., 2017). This observation produces further concerns over the developmental competence of such oocytes. Since cytoskeletal meshwork is crucial for cytokinesis (Li, 2013), loss of its integrity may negatively affect cell cleavage following fertilization. Consistently, fertilization rates and proportions of good quality embryos are lower with SERa+ oocytes compared to those with control oocytes (Rienzi et al., 2008).

The third biological process that was significantly down-regulated in SERa+ oocytes, with respect to normal counterparts, involved mitochondrial structure and respiratory activity, suggesting that SERa may also reflect perturbation of a functional link between the endoplasmic reticulum and the mitochondrial network. The association of endoplasmic reticulum and mitochondria has been well known since the 1960s (Goetz and Nabi, 2006) and subsequent studies have revealed that contact between these organelles is achieved by close juxtaposition via mitochondria-associated membranes (MAM). One of the MAM functions is regulation of calcium transport from the endoplasmic reticulum to mitochondria (Goetz and Nabi, 2006). In SERa+ oocytes, it has been demonstrated that an atypical pattern of calcium release has profound consequences on normal mitochondrial respiratory activity and mitochondrial ATP generation (Hajnóczky et al., 2000; Van Blerkom, 2011). Since the balance of ATP supply by mitochondria is one of the crucial factors in defining fertilization and developmental competence for the oocyte and the embryo, respectively, our gene expression results for the first time demonstrate at the molecular level that in SERa+ oocytes defects in calcium regulation by mitochondria and energytic ATP homeostasis could have negative downstream developmental effects.

On the whole, for the first time, we provide information on the molecular status of SERa+ oocytes, highlighting possible associations between the presence of SERa, altered oocyte physiology and reduced developmental competence. We believe that our study may offer further information that can support embryologists to make decisions on whether, and with what possible implications, SERa+ oocytes should be used for IVF treatments. The presence of SERa should be still a 'red flag' in IVF practices and we agree with the recent Alpha/ESHRE Consensus that the decision to use SERa+ oocytes should be discussed on a case-by-case basis (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Correct counseling about the risk of birth malformations and caution when considering transfers of embryos affected by SERa are mandatory, especially for those couples with a limited number of available SERa− oocytes or with all SERa+ retrieved oocytes, due to the possible negative effects in terms of transfer cancellation. In the meantime, the currently conflicting data require large surveys among centers, recording of clinical outcomes and malformation rates and follow-up of babies born after the transfer of embryos from SERa+ oocytes.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

S.S. performed experiments and contributed to critical discussion. S. M. performed microarray data analyses. I.C. and P.C. contributed to collection of oocytes. V.R. and P.A. contributed to critical discussion. P.S. designed the study, analyzed and interpreted the data, drafted the article and contributed to critical discussion. All authors read and approved the article.

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Conflict of interest

The authors declare no conflict of interest. Ferring Pharmaceuticals had no role in study design, data collection, data analysis, data interpretation or writing of the article.

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